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(54) Title: SOLUBLE PRION POLYPEPTIDES, AND METHODS FOR DETECTING AND PURIFYING THEREOF

(57) Abstract

Novel soluble prion polypeptides occurring *in vivo*, and methods of making and using thereof. Diagnostic methods are also provided having both generality and rapidity for the identification of soluble infectious or normal prion polypeptides in animal or human subjects which provide a diagnosis as to present or future prion-related pathologies, which methods provide relatively simple and commercially useful diagnostic assays which can be applied in the field or in the laboratory for the detection of prion infection in livestock and humans.

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# SOLUBLE PRION POLYPEPTIDES, AND METHODS FOR DETECTING AND PURIFYING THEREOF

## BACKGROUND OF THE INVENTION

### Field of the Invention

The invention in the field of molecular and cell biology relates to novel soluble prion polypeptides occurring *in vivo*, and to methods of making and using thereof.

### Background of Related Art

The cellular prion protein ($PrP^c$) is a sialoglycoprotein encoded by a gene that in humans is located on chromosome 20 (Oesch, B. et al. Cell 40:735-746 (1985); Basler, K. et al. 46:417-428 (1986); Liao, Y.J. et al. Science 233:364-367 (1986); Meyer, R.K. et al. Proc. Natl. Acad. Sci. USA 83:2310-2314 (1986); Robakis, N.K. et al. Biochem. Biophys. Res. Commun. 140:758-765 (1986); Sparkes, R.S. et al. Proc. Natl. Acad. Sci. USA 83:7358-7362 (1986); Bendheim, P.E. et al. J. Infect. Dis. 158:1198-1208 (1988); Turk, E. et al. Eur. J. Biochem. 176:21-30 (1988)). The PrP gene is expressed in neural and non-neural tissues, the highest concentration of mRNA being in neurons (Chesebro, B. et al. Nature 315:331-333 (1985); Kretzschmar, H.A. et al. Am. J. Pathol. 1221-5 (1986); Brown, H.R. et al. Acta Neuropathol. 80:1-6 (1990); Cashman, N.R. et al. Cell 61:185-192 (1990); Bendheim, P.E. Neurology 42:149-156 (1992)).

The translation product of the PrP gene consists of 253 amino acids in humans (Kretzschmar, H.A. et al. DNA 5:315-324 (1986); Pucket, C. et al. Am. J. Hum. 49:320-329 (1991)), 254 in hamster and mice or 256 amino acids in sheep and undergoes several post-translational modifications. In hamsters, a signal peptide of 22 amino acids is cleaved at the N-terminus, 23 amino acids are removed from the C-terminus on addition of a glycosyl phosphatidylinositol (GPI) anchor, and aspargine-linked oligosaccharides are attached to residues 181 and 197 in a loop formed by a disulfide bond (Turk, E. et al. Eur. J. Biochem. 176:21-30 (1988); Hope, J. et al. EMBO J. 5:2591-2597 (1986); Stahl, N. et al. Cell 51:229-240 (1987); Stahl, N. et al. Biochemistry 29:5405-5412 (1990); Safar, J. et al. Proc. Natl. Acad. Sci. USA 87:6377 (1990)).

In prion-related encephalopathies, such as Creutzfeldt-Jakob disease (CJD) and Gerstmann-Sträussler-Scheinker disease humans (GSS) of humans, scrapie of sheep and goats, and spongiform encephalopathy of cattle, $PrP^c$ is converted into an altered form designated $PrP^{Sc}$, that is distinguishable from $PrP^c$ because only the N-terminal 67 amino acids are removed by proteinase K digestion under conditions in which $PrP^c$ is completely degraded (Oesch B. et al. Cell 40:735-746 (1985); Bolton, D.C. et al. Science 218:1309-1311 (1982); McKinley, M.P. et al. Cells 35:57-62 (1982); Bolton, D.C. et al. Biochemistry 23:5898-5905 (1984); Prusiner, S.B. et al. Cell 38:127-134 (1984); Bolton, D.C. et al. Arch. Biochem. Biophys. 258:1515-22 (1987)).

Several lines of evidence suggest that $PrP^{Sc}$ may be a key component of the transmissible agent responsible for prion-related encephalopathies (Prusiner, S.B. Science 252:1515-22 (1991) and it has been established that its protease-resistant core is the major structural protein of amyloid fibrils that accumulate intracerebrally in some of these conditions (Brendheim, P.E. et al. Nature 310:418-421 (1984); DeArmond, S.J. et al. Cell 41:221-235 (1985); Kitamoto, T. et al. Ann. Neurol. 20:204-208 (1986); Robert, G.W. et al. N. Engl. Med. 315:1231-1233 (1986); Ghetti, B. et al. Neurology 39: 1453-1461 (1989); Tagliavini, F. et al. EMBO. J. 10:513-519 (1991); Kitamoto, T. et al. Neurology 41:306-310 (1991)).

$PrP^C$ is a membrane-bound protein, anchored to the cell surface membrane through the GPI moiety (Stahl, N. et al. Cell 51:229-240 (1987); Stahl, N. et al. Biochemistry 29:8879-8884 (1990); Stahl, N. et al. Biochemistry 29:5405-5412 (1990); Safar, j. et al. J. Infec. Dis. 163:488-494 (1991). Nevertheless, a secretory form of the molecule, in addition to the membrane form, has been found in cell-free translation systems supplemented with microsomal membranes.

$PrP^C$ is secreted from Xenopus oocytes injected with PrP mRNA synthesized in vitro (Hay, B. et al. Biochemistry 26:8110-8115 (1987). $PrP^C$ is spontaneously released from normal and scrapie-infected murine neuroblastoma cells, and from mouse C127 cells transfected with the PrP gene cloned from scrapie-infected

mouse brain (Caughey, B. et al. Proc. Natl. Acad. Sci. USA 85:4757-4661 (1988); Caughey, B. et al. J. Virol. 63:175-181 (1989); Borchelt, D.R. et al. J. Cell. Biol. 110:743-752 (1990)). A secretory form of PrP in addition to a transmembrane form can be generated in cell-free translation systems supplemented with microsomal membranes. The secretory form predominates in the rabbit reticulocyte lysate system, whereas the transmembrane form prevails in the wheat germ system, the alterative topology being controlled by a stop-transfer effector domain (Prusiner, S.B. Nature 310:418-421 (1984); Hay, B. et al. Biochemistry 26:8110-8115 (1987); Hay B. et al. Mol. Cell. Biol. 7:914-920 (1987); Lopez, C.d. et al. Science 248:226-229 (1990); Yost, C.S. et al. Nature 343:669-672 (1990)).

Inoculation of CSF from patients with Creutzfeldt-Jakob disease and animals with natural or experimental scrapie induces a spongiform encephalopathy in the recipient animal (Brown, P. Epidemiol Rev. 2:113-135 (1989). Non-GPI-linked, C-terminal-truncated molecules have been isolated from scrapie-infected hamster brains (Stahl, N. et al. Biochemistry 29:8879-8884 (1990). GPI-linked $PrP^{SC}$ accumulates within cytoplasmic vesicles of cultured cells instead of being exported to the plasma membrane (Taraboulos, A. et al. J. Cell. Biol. 110:2117-2132 (1990).

Sequencing studies of C-terminal peptides derived from enzymatic digestion of $PrP^{Sc}$ purified from scrapie-infected hamster brains have shown that approximately 15% of the molecules do not contain the GPI anchor and are truncated at the C-terminus, ending at Gly228 rather than at Ser231 (Stahl, N. et al. Biochemistry 29:8879-8884). It is likely that such truncation at Gly228 renders the prion hamster $PrP^{Sc}$ soluble *in vitro*, but such truncated scrapie proteins have not been found to exist *in vivo* in a substantially soluble form. Soluble forms of any prion proteins have thus not been demonstrated *in vivo* or *in situ*.

Accordingly, the lack of a soluble form of prion polypeptides makes impractical the routine screening of animals or humans for normal or pathogenic prion expression, such that tissues (e.g., as brain) need to be biopsied to determine the

pathogenesis. If prion proteins could be reliably assayed, it could conceivably be decades before neurological symptoms begin to occur. However, as biopsy is the only known means of detection, testing is not provided usually until neurodegenerative symptoms begin.

Therefore, a need exists to provide alternative detection methods for prion proteins in animals or humans for diagnosing present or potential prion related infection or pathogenesis.

## SUMMARY OF THE INVENTION

It is an object of the present invention to overcome the deficiencies of the related art.

It is also an object of the present invention to provide novel diagnostic methods having both generality and rapidity for the identification of soluble prion polypeptides in animal or human subjects which provide a diagnosis as to present or future prion-related pathologies, which methods provide relatively simple and commercially useful diagnostic assays which can be applied in the field or in the laboratory for the detection of prion infection in livestock and humans.

It is also an object of the present invention to provide novel prion polypeptides which can be found *in vivo* which are in substantially soluble form as soluble prion polypeptides which are capable of being detected in body tissues, preferably blood or cerebrospinal fluid.

It is a further object of the present invention to provide methods for detecting soluble prion polypeptides in a sample, such as a tissue sample of an animal or human subject.

Another object of the present invention is to provide a method for purifying a soluble prion polypeptide from a sample, such as a tissue sample from an animal or a human subject.

Another object of the present invention is to provide for the identification of soluble prion polypeptides which are structurally or functionally related to known prion proteins which, however, are not known to be in substantially soluble form *in vivo or in situ*.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 represents an immunoblot analysis of CSF fraction P2p showing a broad band with electrophoretic mobility of 33-37 kDa (arrows) that is strongly labeled by the antiserum anti-PrPN (lane a). The immunoreactivity is abolished by absorption of the antiserum with the relevant peptide (lane b). Protein bands still immunoreactive after absorption were regarded as nonspecific. Molecular weight markers (expressed in kDa) are shown to the left. 15% polyacrylamide minigel; antisera dilutions: 1:500.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

It has now been unexpectedly discovered that prion polypeptides are expressed in soluble form in animal or human body tissues, such as cerebrospinal fluid, so that detection or diagnosis of prion infected or expressing animals or humans can be performed on a commercial and medically significant scale to avoid or prevent further prion infection among animals or humans, such as for livestock inspection prior to slaughtering, and for diagnosis of prion infection in humans or animal subjects.

According to one aspect of the present invention, a soluble prion polypeptide has been discovered which has an amino acid sequence substantially corresponding to a soluble prion protein selected from a $PrP^{C}$, a PrP or a $PrP^{Sc}$ prion protein which are apparent isoforms of a prion protein having different post-translational modifications. *In vivo* soluble forms of these prion proteins were heretofore unknown and unexpected, due to the formation of prion aggregates of the pathogenic PrP and/or $PrP^{Sc}$ forms, as well as the high hydrophobic and/or aggregation proteins of these isoforms, which are known to occur only associated with cell membranes, such as bound to the GPI anchor, or associated with or incorporated into vesicles.

In the context of the present invention, an *in vivo* soluble prion polypeptide refers to a prion polypeptide corresponding to known insoluble prion proteins, but which insoluble prion polypeptide has been rendered soluble such that if it is found intercellularly or intracellularly and not in association with membranes, vesicles, aggregates or

phospholipids. Accordingly, such newly discovered soluble prion polypeptides are found in soluble form *in vivo*, or under physiological conditions, in various tissues, such as the non-limiting examples of cerebrospinal fluid (CSF), blood, serum and lymph.

Accordingly, the present invention resides in the discovery of *in vivo* soluble isoforms or derivatives of prion proteins, which are found to be present in various tissues of animals and humans, and which provide a means for detecting pathogenic or normal isoforms of prion proteins as *in vivo* soluble polypeptides present in human or animal tissues or soluble extracts thereof.

In one embodiment, the present invention is directed to a naturally occurring soluble prion polypeptide substantially free from impurities of human origin with which it is natively associated. "Substantially free of other proteins" indicates that the protein has been purified away from at least 90 per cent (on a weight basis), or from even at least 99 per cent, if desired, of other proteins and glycoproteins with which it is natively associated. Purification of such soluble prion polypeptides of the present invention can be achieved by subjecting the cells, tissue or fluids containing the soluble prion polypeptide to protein purification techniques such as immunoadsorbent columns bearing monoclonal or polyclonal antibodies reactive against the protein. Alternatively, the purification can be achieved by a combination of standard methods, such as ammonium sulfate precipitation, molecular sieve chromatography, and ion exchange chromatography.

It will be understood that a soluble prion polypeptide of the present invention can be purified biochemically or physicochemically from a variety of cell or tissue sources of animals or humans. For preparation of naturally occurring soluble prion polypeptide, tissues such cerebral spinal fluid, blood, urine or lymph are preferred. However, by routine experimentation it can be determinied whether such soluble prion proteins accumulate in any given tissue.

Prion polypeptides of the present invention include polypeptides having amino acid sequences which substantially

correspond to known prion protein sequences, such as those for PrP, $PrP^C$ and $PrP^{Sc}$, of animal or human origin, but which are in substantially *in vivo* soluble form, due to C-terminal truncation, modification or alternative folding after synthesis. In the context of the present invention, the term "C-terminus" of a prion protein refers to amino acids 127-253, and more preferably, amino acids 228-253 of a known prion protein. The amino acid and nucleic acid sequences of non-soluble prion proteins are known in the art, and such published sequences are herein entirely incorporated by reference, including all sequences and figures, which references include, but are not limited to, Oesch et al. Cell, 40:735-746 (1985); Basler et al. Cell, 46:417-428 (1986); Kretzschmar et al DNA, 5:314-324 (1986); Liao et al. Science, 233:364-367 (1986); Locht et al. Proc. Nat'l Acad. Sci. USA, 8:6372-6376 (1986); Goldmann et al. Proc. Nat'l Acad. Sci. USA, 87:2476-2480 (1990); Puckett et al Am. J. Hum. Genet., 49:320-329 (1991). These sequences, as described above, include prion proteins from different mammalian species having substantial homology. All of the prion sequences are commonly numbered 1-253 as the initially translated, and undergo subsequent post-translational modifications to become the various forms, e.g., as normal $PrP^C$ or abnormal or infectious $PrP^{Sc}$ and/or PrP. Human prion sequences are known to have 253 amino acids, which the C-terminus may be cleaved when Ser231 is bound to GPI. Accordingly, the common 253 numbering system described herein will use this 253 amino acid residue designation, and additional amino acid residues, such as 254 in hamster or 256 in sheep will be so designated.

C-terminal modifications of insoluble prion polypeptides of the present invention include, but are not limited to C-terminal truncations selected from a truncation selected from truncation at prion amino acid 228, 229, 230 or 231; prion amino acids 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250 or prion amino acids 251, 252, 253, 254, 255, 256.

The C-terminal modifications which make the insoluble protein *in vivo* soluble also include C-terminal modifications which render the C-terminus incapable of binding a GPI anchor,

wherein the modification may be selected from a modified serine residue; a modified glycine residue, a modified arginine residue or at least one modified amino acid of prion residues 228 to 256 that renders the Ser230 or Ser231 blocked from binding the GPI anchor. Such C-terminal modifications result in a lack of ability to attach to the GPI anchor and/or in conformational changes of the prion polypeptide such that the prion protein is rendered soluble under *in vivo* conditions.

Another group of prion polypeptides which are intended to be encompassed by the present invention, as "substantially corresponding" to amino acid sequences of known prion isoform proteins, are those in which at least one amino acid residue in a soluble prion protein molecule has been removed and a different residue inserted in its place, the number of substitutions being relatively small and well characterized or conservative, as described herein. For a detailed description of protein chemistry and structure, see Schulz, G.E. <u>et al.</u>, <u>Principles of Protein Structure</u>, Springer-Verlag, New York, 1978, and Creighton, T.E., <u>Proteins: Structure and Molecular Properties</u>, W.H. Freeman & Co., San Francisco, 1983, which are hereby incorporated by reference. The types of substitutions which may be made in the protein or peptide molecule of the present invention may be based on analysis of the frequencies of amino acid changes between a homologous protein of different species, such as those presented in Table 1-2 of Schulz <u>et al.</u> (<u>supra</u>) and Figure 3-9 of Creighton (<u>supra</u>). Based on such an analysis, conservative substitutions are defined herein as exchanges within one of the following five groups:

1. Small aliphatic, nonpolar or slightly polar residues: ala, ser, thr (pro, gly);
2. Polar, negatively charged residues and their amides: asp, asn, glu, gln;
3. Polar, positively charged residues: his, arg, lys;
4. Large aliphatic, nonpolar residues: met, leu, ile, val (cys); and
5. Large aromatic residues: phe, tyr, trp.

The three amino acid residues in parentheses above have

special roles in protein architecture. Gly is the only residue lacking any side chain and thus imparts flexibility to the chain. Pro, because of its unusual geometry, tightly constrains the chain. Cys can participate in disulfide bond formation which is important in protein folding. Note that Schulz et al. would merge Groups 1 and 2, above. Note also that tyr, because of its hydrogen bonding potential, has some kinship with ser, thr, etc.

Analogues of soluble prion polypeptides of the present invention include those with substantial changes in functional or immunological properties may be made to prion polypeptides of the present invention by selecting substitutions that are less conservative, such as between, rather than within, the above five groups, which will differ more significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Examples of such substitutions are (a) substitution of gly and/or pro by another amino acid or deletion or insertion of gly or pro; (b) substitution of a hydrophilic residue, e.g., ser or thr, for (or by) a hydrophobic residue, e.g., leu, ile, phe, val or ala; (c) substitution of a cys residue for (or by) any other residue; (d) substitution of a residue having an electropositive side chain, e.g., lys, arg or his, for (or by) a residue having an electronegative charge, e.g., glu or asp; or (e) substitution of a residue having a bulky side chain, e.g., phe, for (or by) a residue not having such a side chain, e.g., gly.

Most deletions, insertions, and substitutions according to the present invention are those which do not produce radical changes in the characteristics of the protein or peptide molecule. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays, either immunoassays or bioassays.

The prion pathogenic activity of a modified prion polypeptide of the present invention can be screened in a suitable screening assay for the desired characteristic. For

example, a change in the immunological character of the protein molecule, such as binding to a given antibody, is measured by a competitive type immunoassay (see below). Biological activity is screened in an appropriate bioassay, such as prion infectivity, as described herein.

Modifications of such peptide properties as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation or the tendency to aggregate with carriers or into multimers, are assayed by methods well known to the ordinarily skilled artisan.

Derivatization with bifunctional agents is useful for cross-linking the peptide to a water-insoluble support matrix or to other macromolecular carriers. Commonly used cross-linking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, Proteins: Structure and Molecule Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and, in some instances, amidation of the C-terminal carboxyl groups.

One non-limiting example of a soluble prion polypeptide according to the present invention is one which has an amino acid sequence that substantially corresponds to the amino acid sequence of a human $PrP^C$ protein, but has a molecular weight of

33-37 kilodaltons on SDS polyacrylamide gel electrophoresis.

The isolated *in vivo* soluble $PrP^C$ prion protein of the present invention may have a C-terminal truncation which provides solubility of a prion protein *in vivo*, under physiolgical conditions, such as physiological pH, ionic strength, tonicity, and the like. In a further preferred embodiment of the present invention, the C-terminal truncation begins after a prion amino acid corresponding to Gly228 of hamster $PrP^{Sc}$. The C-terminal truncation may begin between the a prion amino acid corresponding to Gly228 of hamster $PrP^{Sc}$ and an amino acid corresponding to Ser231 or Ser232 of human $PrP^C$. The truncation may also be between a prion amino acid corresponding to amino acid Gly228 of hamster $PrP^{Sc}$ and a PIPLC digestion site phosphatidyl inositol phospholtipase C, which cleaves at inositol glycan of GPI as the lipid to prevent .

The prion polypeptide of the present invention may also have a prion amino acid, essential for binding to the GPI anchor site, which is modified or deleted such that the prion polypeptide according to the present invention is rendered soluble, and wherein the *in vivo* soluble prion polypeptide has an truncated or non-truncated C-terminus substantially corresponding to a known insoluble prion protein, such that the folding confirmation of the peptide (as a function of one or more modified, deleted or substituted amino acid, or facilitate folding in the presence of a chaperonin or folding protein) renders the prion polypeptide soluble under *in vivo* conditions.

The presence of a soluble prion protein in a sample derived from a tissue of an animal may be detected by contacting the sample, which has undergone at least one of precipitation, centrifugation, dialysis, column chromatography and/or affinity chromatography, with a detectably labeled antibody which binds a C-terminal epitope specific to a soluble prion polypeptide; and detecting the soluble polypeptide which is bound to the labeled antibody. The antibodies can be selected from polyclonal or monoclonal antibodies generated against prion proteins or fragments thereof or the inositol glycan moeity of GPI.

Detection of soluble forms according to the present invention can also be used to determine PrP infection or the

presence of abnormal forms, such as PrP or $PrP^{Sc}$, using proteinase K digestion of the cell or tissue sample containing soluble prion protein, prior to immunodetection, in order to remove the normal soluble form as $PrP^{C}$, since the pathogenic or infectious form of PrP, as PrP or and/or $PrP^{Sc}$ is not subtantially degraded by proteinase K.

To determine whether any given tissue or physiological or waste fluid source is a candidate for detection of soluble prion polypeptides, a sample of such a fluid or tissue is taken from an animal in which a soluble prion protein has already been detected in the cerebrospinal fluid. The same assay may then be performed, or any other suitable assay, on the tissue or fluid in question. If the soluble prion protein is found therein, then the source of such prion protein may be considered to be a source for the detection of a soluble prion protein in any animal. If the results are negative, the source of such a fluid or tissue is not expected to be useful for detection of a soluble prion protein. As indicated above, it is expected that a soluble prion protein will be widely prevalent in the tissues and fluids of aninals. Alternatively, methods are well known for the synthesis of polypeptides of desired sequence on solid phase supports and their subsequent separation from the support. Once the exact sequence of the portion of the prion protein which causes *in vivo* solubility is determined (by routine techniques), it can also be synthesized. As a non-limiting example, such peptides, ot the isolated soluble prion protein itself, can be used for generation of anti-prion polypeptide antibodies which are specific for soluble forms of prion proteins, and which distinguish over the insoluble forms.

The solution sample used for detection can be an animal or human subject tissue sample selected from cerebrospinal fluid, and are expected to also include blood, plasma, lymph, urine, saliva, brain, nervous tissue, eyes, or an internal organ. Animals include cows, sheep, goats, pigs, horses, elk, deer, rodents, mule and mink, and other mammals and birds.

A detection method according to the present invention can be any immunoassay selected from a an antibody capture assay, an antigen capture assay or a two-antibody sandwich assay. Such

immunoassays are well known in the art and an anti-prion antibody of the present invention can be used according to known immunoassay steps. Non-limiting examples of immunoassays suitable for use in detecting methods of the present invention include direct and sandwich ELISAs, radioimmune assays, enzyme immunoassays and the like, which method steps are well known in the art, as further described herein.

The soluble form of infectious or abnormal forms of prion protein are differentiated from normal forms, such as $PrP^{C}$, by digestion with proteinase K of the sample prior to immunoblot analysis. Good results are expected for determining the presence of soluble forms of infectious or abonormal prion proteins using proteinase K digestion prior to immunoscreening using antibodies specific for an epitope located in the prion polypeptide.

This invention is also directed to an antibody specific for an epitope of a prion protein. Preferably antibodies of the present invention are specific for an epitope of at least 4 amino acids corresponding to at least 4 amino acids of residues 23-228, 23-227, 1-227, 1-228, 1-231, 23-231, 127-231, 200-228 and 200-227. The epitope may include, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 20, 25, 30, 40, 45, or more amino acids, as linear residues in the amino acid chain, or as amino acids forming an epitope due to the three-dimentional configuration of the prion protein *in vivo* or *in situ*. In a preferred embodiment, the epitope is a C-terminal epitope N-terminal to an amino acid corresponding to Gly228 of hamster $PrP^{Sc}$. In another preferred embodiment of the present invention, the C-terminal epitope is between a prion amino acid corresponding to Gly228 of hamster $PrP^{Sc}$ and an amino acid corresponding to Ser231 or Ser232 of human $PrP^{C}$. In still another preferred embodiment, the C-terminal epitope is between a prion amino acid corresponding to amino acid Gly228 of hamster $PrP^{Sc}$ and a PIPLC digestion site. In another preferred embodiment, the C-terminal epitope contains a prion amino acid, essential for binding to the GPI anchor site, which is modified or deleted such that the prion polypeptide according to the present invention is rendered soluble, and wherein the *in vivo* soluble prion polypeptide has a truncated or non-truncated N-terminus substantially corresponding to a known insoluble prion

protein, such that the folding confirmation of the peptide (with one or more modified, deleted or substituted amino acids) resulting solublility of the prion protein. Alternatively or additionally, folding in the presence of a chaperonin or folding protein is expected to renders the prion polypeptide soluble under *in vivo* conditions.

In additional embodiments, the antibody of the present invention is used to detect the presence of, or measure the quantity or concentration of, soluble prion proteins in a cell, or in a cell or tissue extract, or a biological fluid.

The term "antibody" is meant to include polyclonal antibodies, monoclonal antibodies (mAbs), chimeric antibodies, and anti-idiotypic (anti-Id) antibodies that are specific for soluble forms of a mammalian prion protein.

An antibody is said to be "capable of binding" a molecule if it is capable of specifically reacting with the molecule to thereby bind the molecule to the antibody. The term "epitope" is meant to refer to that portion of any molecule capable of being bound by an antibody which can also be recognized by that antibody. Epitopes or "antigenic determinants" usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and have specific three dimensional structural characteristics as well as specific charge characteristics.

An "antigen" is a molecule or a portion of a molecule capable of being bound by an antibody which is additionally capable of inducing an animal to produce antibody capable of binding to an epitope of that antigen. An antigen may have one or more than one epitope. The specific reaction referred to above is meant to indicate that the antigen will react, in a highly selective manner, with its corresponding antibody and not with the multitude of other antibodies which may be evoked by other antigens.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen.

Monoclonal antibodies are a substantially homogeneous population of antibodies to specific antigens. MAbs may be ob-

tained by methods known to those skilled in the art. See, for example Kohler and Milstein, Nature 256:495-497 (1975); U.S. Patent No. 4,376,110; Ausubel et al, *eds*. CURRENT PROTCOLS IN MOLECULAR BIOLOGY, Wiley Interscience, New York (1987, 1992); and Harlow and Lane, ANTIBODIES, A LABORATORY MANUAL, Cold Spring Harbor Laboratory (1988), the entire contents of which references are herein incorporated by reference. Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, and any subclass thereof. The hybridoma producing the mAbs of this invention may be cultivated in vitro or in vivo. Production of high titers of mAbs in vivo production makes this the presently preferred method of production. Briefly, cells from the individual hybridomas are injected intraperitoneally into pristane-primed Balb/c mice to produce ascites fluid containing high concentrations of the desired mAbs. MAbs of isotype IgM or IgG may be purified from such ascites fluids, or from culture supernatants, using column chromatography methods well known to those of skill in the art.

Chimeric antibodies are molecules different portions of which are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region. Chimeric antibodies and methods for their production are known in the art (see, for example, Morrison et al., Proc. Natl. Acad. Sci. USA 81:6851-6855 (1984); Neuberger et al., Nature 314:268-270 (1985); Sun et al., Proc. Natl. Acad. Sci. USA 84:214-218 (1987); Better et al., Science 240:1041- 1043 (1988); Better, M.D. International Patent Publication WO 9107494, which references are hereby incorporated by reference).

An anti-idiotypic (anti-Id) antibody is an antibody which recognizes unique determinants generally associated with the antigen-binding site of an antibody. An Id antibody can be prepared by immunizing an animal of the same species and genetic type (e.g. mouse strain) as the source of the mAb with the mAb to which an anti-Id is being prepared. The immunized animal will recognize and respond to the idiotypic determinants of the immunizing antibody by producing an antibody to these idiotypic determinants (the anti-Id antibody).

The anti-Id antibody may also be used as an "immunogen" to induce an immune response in yet another animal, producing a so-called anti-anti-Id antibody. The anti-anti-Id may bear structural similarity to the original mAb which induced the anti-Id. Thus, by using antibodies to the idiotypic determinants of a mAb, it is possible to identify other clones expressing antibodies of identical specificity.

Accordingly, mAbs generated against the prion protein of the present invention may be used to induce anti-Id antibodies in suitable animals, such as Balb/c mice. Spleen cells from such immunized mice are used to produce anti-Id hybridomas secreting anti-Id mAbs. Further, the anti-Id mAbs can be coupled to a carrier such as keyhole limpet hemocyanin (KLH) and used to immunize additional Balb/c mice. Sera from these mice will contain anti-anti-Id antibodies that have the binding properties of the original mAb specific for a prion protein epitope.

The anti-Id mAbs thus have their own idiotypic epitopes, or "idiotopes" structurally similar to the epitope being evaluated, such as an epitope of a prion protein.

The term "antibody" is also meant to include both intact molecules as well as fragments thereof, such as, for example, Fab and $F(ab')_2$, which are capable of binding antigen. Fab and $F(ab')_2$ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact antibody (Wahl et al., J. Nucl. Med. 24:316-325 (1983)).

It will be appreciated that Fab and $F(ab')_2$ and other fragments of the antibodies useful in the present invention may be used for the detection and quantitation of a prion protein according to the methods disclosed herein for intact antibody molecules. Such fragments are typically produced by proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce $F(ab')_2$ fragments).

The antibodies, or fragments of antibodies, of the present invention may be used to quantitatively or qualitatively detect the presence of cells which express a prion protein (or a chimeric receptor having a prion-derived epitope) in soluble form. This can be accomplished by immunofluorescence techniques

employing a fluorescently labeled antibody (see below) coupled with light microscopic, flow cytometric, or fluorometric detection.

The antibodies of the present invention may be employed histologically, as in immunofluorescence or immunoelectron microscopy, for in situ detection of a prion protein. In situ detection may be accomplished by removing a histological (cell or tissue) specimen from a subject and providing the a labeled antibody of the present invention to such a specimen. The antibody (or fragment) is preferably provided by applying or by overlaying on the biological sample. Through the use of such a procedure, it is possible to determine not only the presence of a prion protein but also its distribution on the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such in situ detection.

Additionally, the antibody of the present invention can be used to detect the presence of soluble prion molecules in a biological sample.

Such immunoassays for prion protein typically comprise incubating a biological sample, such as a biological fluid, a tissue extract, or freshly harvested cells, from such sources as CSF, or other body tissues, fluids, or waste fluids, or extracts or derivatives thereof, or cells which have been incubated in tissue culture, in the presence of a detectably labeled antibody capable of identifying soluble prion protein, and detecting the antibody by any of a number of techniques well-known in the art.

A soluble prion protein may be detected using polyclonal or monoclonal antibodies specific for insoluble or soluble prion proteins, since the insoluble prion proteins are not expected to be present in body fluids such as CSF or blood. However, monoclonal antibodies which distinguish between soluble and insoluble prion protein are also part of the present invention, since the discovery and isolation of soluble prion proteins allows for the generation of monoclonal antibodies according to known method steps, from which soluble specific monoclonals can be routinely screened using known method steps

to isolate soluble prion specific monoclonal antibodies that do not bind to insoluble forms of prion proteins.

The biological sample may be treated with a solid phase support or carrier (which terms are used interchangeably herein) such as nitrocellulose, or other solid support which is capable of immobilizing cells, cell particles or soluble proteins. The support may then be washed with suitable buffers followed by treatment with the detectably labeled prion-specific antibody. The solid phase support may then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on the solid support may then be detected by conventional means.

By "solid phase support" or "carrier" is intended any support capable of binding antigen or antibodies. Well-known supports, or carriers, include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, gabbros, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Those skilled in the art will know many other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

The binding activity of a given lot of anti-prion antibody may be determined according to well-known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

One of the ways in which the antibody can be detectably labeled is by providing peptide probes or anti-target protein antibodies and linking the peptide probes or antibodies to an enzyme and use in an enzyme immunoassay (EIA). This enzyme, in turn, when later exposed to an appropriate substrate, will react

with the substrate in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorometric or by visual means. Enzymes which can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. The detection can be accomplished by colorimetric methods which employ a chromogenic substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

Detection may be accomplished using any of a variety of other immunoassays or detectably labeled anti-prion antibodies or antibodies that bind anti-prion antibodies. For example, by radioactively labeling the anti-prion antibodies, antibody fragments, or antibodies specific therefore, it is possible to detect the labeled target protein through the use of a radioimmunoassay (RIA). A good description of RIA may be found in Laboratory Techniques and Biochemistry in Molecular Biology, by Work, T.S., et al., North Holland Publishing Company, New York (1978) with particular reference to the chapter entitled "An Introduction to Radioimmune Assay and Related Techniques" by T. Chard, incorporated by reference herein. The radioactive isotope can be detected by such means as the use of a gamma counter or a liquid scintillation counter or by autoradiography.

It is also possible to label anti-prion antibodies, antibody fragments, or antibodies specific therefore, with a fluorescent compound. When the fluorescently labeled anti-prion antibodies, antibody fragments, or antibodies specific therefore, is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labelling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

Anti-prion antibodies, antibody fragments, or anti-idiotype antibodies specific therefore, can also be detectably labeled using fluorescence emitting metals such as $^{152}Eu$, or others of the lanthanide series. These metals can be attached to the anti-prion antibodies, antibody fragments, or anti-idiotype antibodies specific therefore, using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The anti-prion antibodies, antibody fragments, or anti-idiotype antibodies specific therefore, also can be detectably labeled by coupling to a chemiluminescent compound. The presence of the chemiluminescent-tagged anti-prion antibodies, antibody fragments, or anti-idiotype antibodies specific therefore, is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the anti-prion antibodies, antibody fragments, or anti-idiotype antibodies specific therefore, of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic anti-prion antibodies, antibody fragments, or anti-idiotype antibodies specific therefore, increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent anti-prion antibodies, antibody fragments, or anti-idiotype antibodies specific therefore, is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

The antibody molecules of the present invention may be adapted for utilization in an immunometric assay, also known as a "two-site" or "sandwich" assay. In a typical immunometric assay, a quantity of unlabeled antibody (or fragment of antibody) is bound to a solid support and a quantity of detectably labeled soluble antibody is added to permit detection and/or quantitation of the ternary complex formed between solid-phase antibody, antigen, and labeled antibody.

Typical, and preferred, immunometric assays include "forward" assays in which the antibody bound to the solid phase is first contacted with the sample being tested to "extract" the antigen from the sample by formation of a binary solid phase antibody-antigen complex. After a suitable incubation period, the solid support is washed to remove the residue of the fluid sample (as a solubilized or aqueous tissue), including antigen (as unreacted prion polypeptide or a fragment thereof), if any, and then contacted with the solution containing an unknown quantity of labeled antibody (which functions as a "reporter molecule"). After a second incubation period to permit the labeled antibody to complex with the antigen bound to the solid support through the unlabeled antibody, the solid support is washed a second time to remove the unreacted labeled antibody.

In another type of "sandwich" assay, which may also be useful with the antigens of the present invention as soluble prion polypeptides, the so-called "simultaneous" and "reverse" assays are used. A simultaneous assay involves a single incubation step as the antibody bound to the solid support and labeled antibody are both added to the sample being tested at the same time. After the incubation is completed, the solid support is washed to remove the residue of fluid sample and uncomplexed labeled antibody. The presence of labeled antibody associated with the solid support is then determined as it would be in a conventional "forward" sandwich assay.

In the "reverse" assay, stepwise addition first of a solution of labeled anti-prion antibody to the fluid sample followed by the addition of unlabeled antibody bound to a solid support after a suitable incubation period is utilized. After a second incubation, the solid phase is washed in conventional fashion to free it of the residue of the sample being tested and the solution of unreacted labeled antibody. The determination of labeled antibody associated with a solid support is then determined as in the "simultaneous" and "forward" assays.

According to the present invention, it is possible to detect soluble prion polypeptides in a tissue of an animal or human subject. This is accomplished by means of an immunoassay, as described above, using an anti-prion antibody of the present

invention or a functional derivative thereof.

According to another aspect of the present invention, known method steps can be used to purify soluble forms of prion proteins, based on the finding that such soluble forms exist *in vivo* and *in situ*, which purification would be obtainable by one ordinary skill in the art using known method steps based on the teaching and guidance presented herein. One non-limiting example of a soluble prion purification method according to the present invention from a solution sample comprises precipitating the sample with ammonium sulfate to provide incremental percentage fractions; centrifuging and dialyzing the fractions and determining which fractions contain soluble prion polypeptides using anti-prion antibodies which bind a prion protein specific epitope, as specific percent fraction which contain the soluble polypeptide; and recovering the soluble polypeptide from the percent fraction containing the *in vivo* soluble prion polypeptide.

Purification of soluble forms or prion proteins according to the present invention can also be used to selectively provide soluble infectious or abnormal prion forms, such as PrP or $PrP^{Sc}$, using proteinase K digestion of the cell or tissue sample containing soluble prion protein, prior to precipitation, in order to remove the normal soluble form as $PrP^{C}$, since the pathogenic or infectious form of PrP, as PrP or and/or $PrP^{Sc}$ is not subtantially degraded by proteinase K.

Preferably, the percent ammonium sulfate fraction is a 30-60% fraction, such as a 40, 45, 50, 55 or 60%. In another preferred embodiment, the antibodies are selected from polyclonal or monoclonal, and are alternatively generated against at least a portion of a PrP, $PrP^{C}$, or a $PrP^{Sc}$ prion polypeptide, as described herein, as a animal or human PrP. Non-limiting examples of animal prions may include those of bovine, ovine, goat, sheep, horse, mouse, hamster, deer, elk, mule and mink, such as those involved in scrapie, spongiform encephalopathy, transmissible mink encephalopathy and chronic wasting disease of mule deer and elk. Human prion related diseases include, but at not limited to Kuru, Creutzfeldt-Jakob Disease (CJD), Gerstmann-Stäussler-Scheinker syndrome (GSS), as well as prion associated

human neurodegenerative diseases.

A soluble prion polypeptide detecting method of the present invention may be used on a sample taken from an animal or human subject tissue selected from cerebral spinal fluid, blood, plasma, lymph, urine, saliva, brain, nervous tissue, eyes, or internal organ.

The following examples are presented by way of explanation and not by way of limitation. The present invention lies in the discovery of soluble prion proteins, and polypeptide, antibodies and purification and detection methods involving these soluble prions based on this discovery are all part of the present invention.

**EXAMPLE 1: DETECTION AND SEQUENCING OF A SOLUBLE HUMAN PRION POLYPEPTIDE IN HUMAN CSF**

MATERIALS AND METHODS: Antisera: Two antisera, designated anti-PrP N were raised in rabbits to the synthetic peptide K-K-R-P-K-P-G-G-W-N-T-G-G-S-R-Y-P-G-G-C (SEQ ID NO:2), that corresponds to residues 23-40 of the amino acid sequence deduced from the human PrP cDNA, except for the addition of Gly-Cys at the C-terminus for spacing and coupling. This peptide was synthesized by solid phase techniques using Fmoc-t-butyl-polyamide chemistry, purified by HPLC using a $\mu$-Bondapak C18 column (Waters), and coupled to keyhole limpet hemocyanin with m-maleimidobenzoyl-N-hydroxysuccinimide. Immunization was carried out according to known method steps, e.g., as described previously (Ghiso et al. Biochem. Biophys. Res. Comm. 163:430-437 (1989)) and antibody liter was evaluated by ELISA. Anti-PrP 27-30 was generated by immunizing rabbits with the protease-resistant core of $PrP^{Sc}$ (i.e., 27-30) purified from scrapie-infected hamster brains (see, Turk et al Eur. J. Biochem. 176:21-30 (1988); Bary and Prusiner J. Infect. Dis. 154:518-521 (1986)).

Fractionation of CSF proteins: Samples of CSF were collected from three individuals aged 3, 17 and 29 years, who received external shunting for tension hydrocephalus due to germinoma of the pineal region, stenosis of the aqueduct and gangliocytoma of the cerebellum, respectively. The protein content of the samples was lower than 100 mg per 100 ml, and

blood cells were absent. By ammonium sulphate precipitation four fractions were obtained, that corresponded to the precipitates at salt concentration of 25%, 50% and 70% (designated as P1, P2 and P3, respectively) and to the 70% supernatant (designated as S3). P1, P2 and P3 were redissolved in 20 mM Tris-HCl, pH 7.5, and dialyzed against distilled water using tubular membranes with a molecular weight cut off of 12,000-14,000. After centrifugation at 50,000 x g for 30 minutes, pellets (designated as P1p, P2p, and P3p, respectively) and supernatants (designated as P1s, P2s and P3s, respectively) were lyophilized. S3 was dialyzed against distilled water and lyophilized.

Immunoblot analysis: Aliquots of the fractions were redissolved in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5, and proteins were determined using the Coomassie blue dye-binding assay (Bio-Rad). Samples containing 50 μg protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% or 15% monomer) under reducing conditions and electrotransferred to ProBlott membrane (Applied Biosystems) using 10 mM 3-cyclohexylamino-1-propanesulfonic acid buffer, pH 11, 10% methanol. The membranes were either stained with Coomassie blue or immunostained with anti-PrPN antiserum (1: 500), as described previously (Tagliavini et al EMBO. J. 10:513-519 (1991)). Proteins specifically detected by the antiserum were identified by using anti-PrPN preabsorbed with 10 mM of the relevant peptide for 60 minutes at 37°C. Aliquots of the CSF fraction containing anti-PrPN immunoreactive proteins were treated with proteinase K (20 μg/ml) for 60 minutes at 37°C. Digestion was terminated by the addition of 20 mM phenylmethylsulfony fluoride, and the samples were subjected to immunoblot analysis with anti-PrP 27-30 antiserum (1:1,000).

Protein Sequence Analysis: Coomassie blue-stained bands corresponding to bands specifically labeled by anti-PrPN antiserum were excised and analyzed with 477A microsequencer (Applied Biosystems) for N-terminal amino sequence. The resulting phenylthiohydanoin amino acid derivatives were identified using the on-line 120A PTH analyzer and the standard program (Applied Biosystems).

Results and Discussion: Immunoblot analysis of CSF

showed that fraction P2, corresponding to the precipitate at 50% ammonium sulfate concentration, contained at 33-37 kDa protein band that was specifically labeled by anti-PrPN antiserum (Figure 1). This band was consistently observed in CSF samples of all subjects and was completely degraded by proteinase K digestion, thus resembling $PrP^C$. N-terminal sequence analysis of the anti-PrP immunoreactive band by automated Edman degradation yielded K-X-P-K-(P)-G-G-(P)-N-T (SEQ ID NO:1), which aligns at position 24 of the amino acid sequence deduced from human PrP cDNA, one residue beyond the predicted signal peptide cleavage site (Puckett, C. et al. Am. J. Hum. Genet. 49:320-329 (1991); Hope, J. et al. EMBO J. 5:2591-2597 (1986)). In this regard, N-terminal heterogeneity of $PrP^{Sc}$ purified from scrapie-infected hamster brains with a major sequence starting at position 23 and two minor sequences beginning at residues 24 and 31, respectively, has been reported (Turk et al Eur. J. Biochem. 176:21-30 (1988)). In the second cycle of the Edman degradation, the Arg signal was not detectded at this position as has been observed previously in sequencing studies of hamster $PrP^C$ and $PrP^{SC}$, suggesting that this residue might undergo post-translational modifications which preclude its detection during gas-phase sequencing (Turk, id.). N-terminal sequences of apolipoproteins E and J, and of the α-chain of complement C4 were also identified in CSF fraction P2p, comigrating with $PrP^C$. These proteins comprised mainly the higher molecular weight sector of the 33-37 kDa protein band, whereas $PrP^C$ was found predominantly in the lower portion of the band.

*In situ* hybridization studies have shown that in the central nervous system PrP mRNA is expressed in a variety of cells, including choroid plexus epithelial cells, ependymal cells and meningeal cells (Brown, H.R. et al. Acta Neuropathol. 80:1-6 (1990)). Accordingly, $PrP^C$ secreted in human CSF is expected to originate from different cell populations. It is expected that $PrP^C$ and/or $Prp^{Sc}$, and other PrP pathogenic prion proteins from animals and humans are derived from membrane-bound molecules by endogenous GPI-releasing activities, correspond to a C-terminal truncated $PrP^C$ derivative, and/or are due to folding proteins such as chaperonins. The soluble form of infectious or

abnormal forms of prion protein are differentiated from normal forms, such as $PrP^C$, by digestion with proteinase K of the sample prior to immunoblot analysis. Good results are expected for determining the presence of soluble forms of infectious or abonormal prion proteins using proteinase K digestion prior to immunoscreening using antibodies specific for an epitope located in the prion polypeptide, preferably for an epitope of at least 3 amino acids corresponding to at least 3 amino acids of residues 23-227, 1-227, 1-228, 1-231, 23-231, 127-231, 200-228 and 200-227.

It is expected that since a soluble form of $PrP^C$ is found in CSF of normal individuals, a soluble form of $PrP^{SC}$ is generated in prion-related encephalopathies. Accordingly, it is expected that PrPSc secreted from cells plays a crucial role in the dissemination of the disease process.

All references cited herein, including journal articles or abstracts, published or corresponding U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the contents of the references cited within the references cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional methods steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the generic concept of the present invention. Therefore, such adaptations and modifications are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance

presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: TAGLIAVINI, FABRIZIO
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(ii) TITLE OF INVENTION: SOLUBLE PRION POLPYPEPTIDES, AMD METHODS FOR DETECTING AND PURIFYING THEREOF

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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(A) APPLICATION NUMBER: US 07/883,875
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(viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Lys Xaa Pro Lys Pro Gly Gly Pro Asn Thr
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Lys Lys Arg Pro Lys Pro Gly Gly Trp Asn Thr Gly Gly Ser Arg Tyr
1               5                   10                      15

Pro Gly Gly Cys
            20
```

WHAT IS CLAIMED IS:

1. A soluble prion polypeptide comprising an amino acid sequence capable of being purified from cerebrospinal fluid.

2. A soluble prion protein, comprising an amino acid sequence substantially corresponding to a peptide sequence of an prion protein selected from post-transcriptionally modified $PrP^C$, a PrP or a $PrP^{SC}$ prion protein, wherein said soluble prion polypeptide has a modified C-terminus such that said polypeptide is soluble under *in vivo* conditions.

3. A soluble prion polypeptide according to claim 1, wherein said prion protein has a molecular weight of 33-37 kilodaltons on SDS polyacrylamide gel electrophoresis and wherein the prion protein comprises the N-terminal amino acid sequence (SEQ ID NO:1) K-X-P-K-P-G-G-P-N-T.

4. A soluble prion polypeptide according to claim 2, wherein said C-terminal modification is selected from a C-terminal truncation of amino acids 228-253 or C-terminal modification.

5. A soluble prion polypeptide according to claim 4, wherein said C-terminal modification is an amino acid modification which renders the C-terminus incapable of binding a GPI anchor, said modification selected from (a) a modified serine residue; (b) a modified arginine residue (c) a modified glycine residue or (d) at least one modified amino acid of prion residues 228 to 256 that renders the Ser230 or Ser231 blocked from binding the GPI anchor.

6. A method for detecting, in a sample, a soluble prion polypeptide according to claim 1, comprising

(A) contacting said sample with a detectably labeled antibody which binds a prion specific epitope; and

(B) detecting any soluble polypeptide which is bound to said labeled antibody.

7. A method according to claim 6, further comprising, prior to said contacting step (a), adding proteinase K to said sample such that $PrP^C$ in said sample is substantially digested and PrP or $PrP^{Sc}$ is substantially undigested.

8. A method according to claim 6, wherein said antibodies are selected from polyclonal or monoclonal.

9. A method according to claim 6, wherein said solution sample is an animal or human subject tissue sample selected from cerebrospinal fluid, blood, plasma, lymph, urine or saliva.

10. A method according to claim 6, wherein said contacting is performed as part of an immunoassay method selected from a an antibody capture assay, an antigen capture assay or a two-antibody sandwich assay, selected from an ELISA, an EIA, a dot blot or an RIA.

11. A method according to claim 6, wherein said detectable labeled antibody is labeled with a detectable label selected from a radiolabel, a fluorescent label and an enzymatic label.

12. A method for purifying a soluble prion polypeptide according to claim 1 from a solution sample, comprising

(A) precipitating said sample with ammonium sulfate to provide incremental percentage fractions;

(B) precipitating and dialyzing the incremental fractions;

(C) contacting the incremental fractions with anti-prion antibodies which bind a prion protein specific epitope to determine a percent fraction which contain said soluble polypeptide; and

(D) recovering said soluble polypeptide from said percent fraction containing said soluble prion polypeptide.

13. A method according to claim 12, further comprising, prior to said precipitating step (a), adding proteinase K to said sample such that $PrP^{C}$ in said sample is substantially digested and PrP or $PrP^{Sc}$ is substantially undigested.

14. A method according to claim 12, wherein said percent fraction is a 50% ammonium sulfate fraction.

15. A method according to claim 13, wherein said antibodies are selected from polyclonal or monoclonal.

16. A method according to claim 12, wherein said solution sample is an animal or human subject tissue sample selected from cerebrospinal fluid, blood, plasma, lymph, urine, saliva, brain, nervous tissue, eyes, or internal organ.

17. An antibody, anti-idiotype antibody or a fragment thereof, comprising an epitope binding portion of an antibody which binds an epitope of a soluble prion polypeptide according to claim 1.

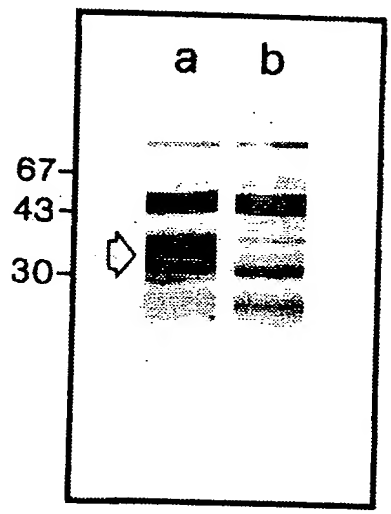
18. An antibody or fragment according to claim 16, wherein said antibody or fragment is monoclonal or polyclonal.

19. An antibody or fragment according to claim 16, wherein said antibody or fragment does not bind an epitope of an insoluble form of a prion protein.

20. An antibody or fragment according to claim 18, wherein said epitope consists essentially of at least 4 amino acids corresponding to amino acids N-terminal to amino acid 228 of a prion protein.

FIG. 1

CSF fraction P2p




a: anti-PrP*N*

b: anti-PrP*N* after absorption

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/04600

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) :C07K 13/00, 15/12, 15/28; C12Q 1/02
US CL :530/395, 388.2; 435/7.92
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/395, 388.2; 435/7.92, 69.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Keyword databases: Dialog, USPTO-APS
Search terms: prion, cerebrospinal
Sequence databases: GenBank, EMBL, GeneSeq, SwissProt, PIR

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>Y | Biochemistry, Volume 26, issued 1987, B. Hay et al., "Evidence for a Secretory Form of the Cellular Prion Protein", pages 8110-8115, especially page 8110 and Figs. 1 and 7. | 1,2,4,5<br>3, 6-20 |
| Y | Biochemistry, Volume 29, Number 38, issued 25 September 1990, N. Stahl et al., "Identification of Glycoinositol Phospholipid Linked and Truncated Forms of the Scrapie Prion Protein", pages 8879-8884, especially the abstract and pages 8882-8883. | 1-20 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 06 August 1993 | AUG 20 1993 |

| Name and mailing address of the ISA/US | Authorized officer |
|---|---|
| Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231 | ROBERT J. HILL, JR. |
| Facsimile No. NOT APPLICABLE | Telephone No. (703) 308-0196 |

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | European Journal of Biochemistry, Volume 176, issued 1988, E. Turk et al., "Purification and properties of the cellular and scrapie hamster prion proteins", pages 21-30, especially the abstract and Fig. 8. | 5-20 |
| X,P | Biochemical and Biophysical Research Communications, Volume 184, Number 3, issued 15 May 1992, F. Tagliavini et al., "A soluble form of prion protein in human cerebrospinal fluid: Implications for prion-related encephalopathies", pages 1398-1404, see the entire document. | 1-20 |
| A | Science, Volume 252, issued 14 June 1991, S. B. Prusiner, "Molecular Biology of Prion Diseases", pages 1515-1522. | 1-20 |
| A | Cell, Volume 38, issued August 1984, S. B. Prusiner et al., "Purification and Structural Studies of a Major Scrapie Prion Protein", pages 127-134. | 1-20 |
| A,P | Biochemistry, Volume 32, issued 1993, D. A. Harris et al., "Processing of a Cellular Prion Protein: Identification of N- and C-Terminal Cleavage Sites", pages 1009-1016. | 1-20 |